Cloning and Primary Structural Analysis of the Bullous Pemphigoid Autoantigen BP180

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Bullous pemphigoid (BP) is an autoimmune skin disease that is characterized by the presence of subepidermal blisters resulting from a disruption of the adhesive interactions between basal keratinocytes and the cutaneous basement membrane. Autoantibodies from patients suffering from this disorder recognize two epidermal antigens, BP180 and BP230, both of which have been localized to the hemidesmosome, a transmembrane structure of stratified, squamous epithelia that functions in cell-matrix adhesion. In the present study we report the primary structural analysis of BP180 based on the sequence of a series of overlapping cDNA clones encompassing 4,669 bases of the BP180 transcript. A polymerase chain reaction-based protocol was used to confirm the contiguity of the cDNA segments. This cloned portion of the BP180 transcript was found to contain one long open reading frame (ORF) 4,596 bases in length. This ORF encodes a polypeptide of 155,000 Daltons with an isoelectric point of 9.7. The carboxyl-terminal half of BP180, a stretch of 916 amino acids, consists of 15 collagen domains of variable length (15 to 242 amino acids) that are separated from one another by short stretches of non-collagen sequences. Located 76 amino acids upstream of the collagenous region is a putative transmembrane domain, a structural feature that distinguishes BP180 from all of the well-characterized members of the collagen family. This membrane-spanning domain is predicted to function as a signal-anchor sequence, directing the C-terminal collagenous segment of this protein to the exterior of the cell. The putative intracellular domain is highly basic with an isoelectric point of 10.37. This molecular analysis predicts that the BP180 antigen is an integral membrane protein of the hemidesmosome that contains a long extracellular collagenous tail. This combination of structural features suggests that BP180 may function as a cell-matrix adhesion molecule, with the collagenous region acting as a potential site of interaction with basement membrane components. Autoantibody-mediated disruption of such an adhesive interaction may play a critical role in the development of sub-epidermal blisters in BP patients. *J Invest Dermatol 99:243–250, 1992*

The clinico-pathologic features of Bullous Pemphigoid (BP), which were first described by Lever in 1952 [1], include tense, sub-epidermal bullae usually associated with an inflammatory infiltrate. Jordon and coworkers [2,3], using immunofluorescence (IF) techniques, demonstrated the presence of circulating and tissue-bound anti-basement membrane zone (BMZ) autoantibodies in BP patients. The antigenic targets of the circulating BP autoantibodies were ultrastructurally localized to the epidermal hemidesmosome [4–7], a membrane-spanning cellular structure thought to be an essential component of the dermal-epidermal adhesion system. Electron microscopic studies have shown BP blister formation is associated with a fragmentation and disappearance of both basal lamina and hemidesmosomal structures [8,9]. Taken together, these observations suggest that the BP humoral autoimmune response directed against a hemidesmosomal antigen may play a key role in the pathogenesis of this blistering skin disorder. It is important to note, however, that direct evidence of the pathogenicity of these anti-hemidesmosomal autoantibodies is lacking.

Immunoblot and immunoprecipitation analyses have demonstrated that two large epidermal polypeptides, BP230 and BP180, are the major antigenic targets of BP autoantibodies [10–12]. Recent studies dealing with the characterization of these autoantigens have employed a molecular genetic approach involving the identification of BP antigen cDNA clones from human and mouse keratinocyte cDNA expression libraries with the use of BP autoantibodies [13–16]. These molecular analyses have resolved the question concerning the biosynthetic relationship between these polypeptides, demonstrating that BP180 and BP230 are the products of distinct and unrelated genes. Complementary DNA clones corresponding to these two antigens hybridized with distinct epidermal RNA transcripts and exhibited no cross-hybridization [15]. Human or rabbit

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Abbreviations:
BP: bullous pemphigoid
BSA: bovine serum albumin
EDTA: ethylenediamine tetraacetic acid
ORF: open reading frame
PCR: polymerase chain reaction
PMSF: phenylmethylsulfonyl fluoride

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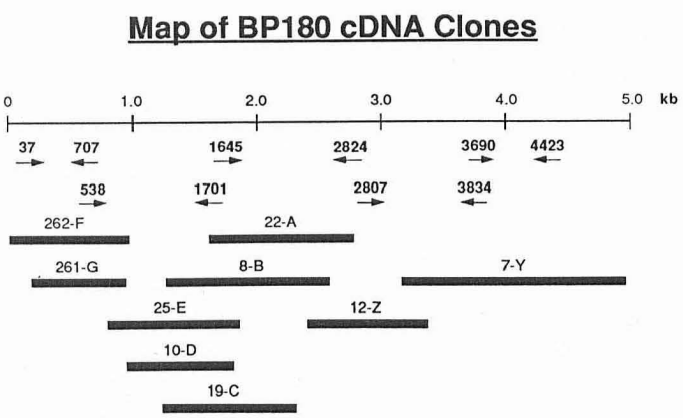

Figure 1. A map representing the sizes and relative positions of the BP180 cDNA clones. These cDNA clones were isolated from two independent human keratinocyte libraries as described in *Materials and Methods*. The first BP180 clone to be isolated, clone 22-A, was identified by antibody screening using a BP serum. Overlapping cDNA clones, represented by the *bars*, were subsequently identified by sequential nucleic acid screening. *Arrows*, positions and the 5′ to 3′ orientation of the oligonucleotide primers (18-mers) used in the PCR analysis presented in Fig 2. The lengths of the *arrows* are not representative of the sizes of the primers.

antibodies against cloned epitopes were shown to exhibit no cross-reactivity between BP230 and BP180 [15]. Chromosomal mapping studies localized the BP230 gene to the short arm of chromosome 6, locus 6p11–12 [17] and the BP180 gene to the long arm of chromosome 10, locus 10q24.3 [18]. Finally, comparison of the full-length BP230 sequence [19] with the BP180 sequence presented in this report has failed to show any significant similarities.

Ultrastructural localization studies using either rabbit antisera or human autoantibody preparations specifically directed against one or the other of these two major BP antigens have shown that both proteins are components of the epidermal hemidesmosome [15,16,20,21]. Both proteins appear to have intracellular domains that are restricted to the hemidesmosomal plaque. Other data have suggested that BP180 may, in addition, have an extracellular domain [22]. The present report provides primary structural evidence that BP180 is a transmembrane protein with a predicted type II orientation — the C-terminus positioned externally. The C-terminal ectodomain consists of a series of collagen triple helical domains which may function in cell-matrix interactions.

MATERIALS AND METHODS

Isolation and Characterization of BP180 cDNA Clones As described in a previous report [15], a high-titer BP serum, BP-R1, was used to identify a 1.0-kb BP180 cDNA clone, λBP180-22A, from an oligo dT-primed, human keratinocyte λgt11 cDNA library (Clontech Laboratories, Palo Alto, CA). A series of overlapping BP180 cDNA clones (Fig 1) were isolated from the library described above (clones 12-Z and 7-Y), or from a random-primed, human keratinocyte λgt11 cDNA library (Clontech Laboratories, Palo Alto, CA) (clones 8B, 19-C, 10-D, 25-E, 261-G, and 262-F) by sequential screening with cRNA probes.

DNA Subcloning and Sequence Analysis Two methods were used for the subcloning of BP180 cDNA into a plasmid vector. The first method involved the conventional ligation of the EcoRI inserts of the λgt11-BP180 clones into the EcoRI site of the pBluescript IISK$^+$ vector (Stratagene, La Jolla, CA). The second method involved polymerase chain reaction (PCR) [23] amplification of a segment of the BP180 cDNA insert using a combination of one BP180-specific primer and one λgt11 vector primer (forward or reverse). The PCR product was subsequently ligated into the cloning site of pCR-1000 using the TA cloning kit (Invitrogen, San Diego, CA). The cDNA inserts were then analyzed according to the double-stranded DNA sequencing procedure of Chen and Seeburg [24], which is a modification of the dideoxy method of Sanger et al [25]. A series of oligonucleotide sequencing primers were synthesized either by the Protein and Nucleic Acid Shared Facility at the Medical College of Wisconsin, or by Operon Technologies, Inc., Alameda, CA. The sequence data were analyzed using the Genetics Computer Group (GCG) sequence analysis software package [26]. Homology searches were run against the GenBank and EMBL nucleotide sequence databases and the Swiss protein sequence database.

Polymerase Chain Reaction PCR [23] was used in the amplification of BP180 cDNA to aid in the subcloning of these DNA segments from the λgt11 bacteriophage vector to a bacterial plasmid vector and to verify that the series of BP180 cDNA clones presented in this report is derived from a single human keratinocyte transcript. For subcloning purposes, the PCR reaction mixture consisted of the following components: BP180-positive λgt11 bacteriophage suspensions from a primary library screen [approximately 4×10^5 pfu in 2 μl of SM solution (100 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatin, 50 mM Tris, pH 7.5)], 20 ng of either the λgt11 forward or reverse primer (Promega Corp., Madison, WI), 20 ng of a BP180-specific oligodeoxynucleotide (18-mer) primer, 100 μM of each deoxynucleotide, 1 unit of Taq DNA polymerase and Taq polymerase buffer (enzyme and 10 × buffer from Promega Corp., Madison, WI), with a final volume of 50 μl. Each PCR reaction mixture was overlaid with 70 μl of mineral oil and subjected to 30 cycles of PCR (94°C, 2 min, 48°C, 2 min, 72°C, 1.5 min) using a Coy Model 60 tempcycler (Coy Corp., Grass Lake, MI). The PCR products were fractionated by agarose gel electrophoresis and visualized by ethidium bromide staining. To verify the contiguity of the BP180 cDNA clones shown in Fig 1, combinations of BP180-specific primers (as described in the legend of Fig 2) were used to amplify segments of BP180 cDNA from a λgt11 human keratinocyte library (a generous gift from Dr. J. Uitto, Philadelphia, PA). This library was constructed independent of the two cDNA libraries from which the set of overlapping BP180 clones were isolated. The PCR products were characterized as described above.

Collagenase Digestion of BP180 from Human Epidermal Extracts Epidermal extracts were treated with collagenase and analyzed according to a modification of the protocol described by Yoshiike et al [27]. Epidermis obtained by keratectomy (6 cm^2) was ground to a powder with a mortar and pestle under liquid nitrogen. The tissue fragments were extracted in 1 ml of a solution containing 150 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol, 62.5 mM Tris, pH 7.4, 0.5 mM PMSF, 7 μg/ml leupeptin, 7 μg/ml antipain, 14 μg/ml pepstatin A, and 14 μg/ml chymostatin. After brief sonication, 25-μl aliquots of the epidermal suspension were pelleted and resuspended in 25 μl of TBSC [0.2 M NaCl, 10 mM $CaCl_2$, 25 mM Tris, pH 7.4, 0.5 mM PMSF, 7 μg/ml leupeptin, 7 μg/ml antipain, 14 μg/ml pepstatin A, and 14 μg/ml chymostatin], with or without 1 BTC unit of collagenase from *Clostridium histolyticum* (Form III; Advance Biofactures Corp., Lynbrook, NY). After incubation at 37°C for various amounts of time, as indicated in the text, the reactions were stopped by incubating at 100°C for 5 minutes in 1× SDS gel sample buffer, then assayed by SDS-PAGE and immunoblotting. A variety of controls were run to test the specificity of the collagenase preparation. PAGE analysis of the epidermal extracts revealed no evidence that any of the major Coomassie Blue-staining proteins were degraded by the collagenase treatment. Immunoblot analysis demonstrated that the BP230 epidermal antigen (a non-collagenous protein) was not degraded by the collagenase. Aliquots of calf skin collagen (type I, Sigma, St. Louis, MO), bovine muscle myosin (Sigma, St. Louis, MO) and bovine serum albumin (BSA; ICN ImmunoBiologicals, Lisle, IL) were incubated for 2 h at 37°C with 1 BTC unit of collagenase. PAGE analysis revealed complete digestion of the calf skin collagen, whereas the myosin and BSA showed no detectable degradation. EDTA inhibition of the collagenase digestions provided additional evidence that the observed degradation of BP180 was due to the action of the well-char-

acterized Ca^{++}-dependent bacterial collagenase, and not to contaminating proteolytic activity.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting Procedures The SDS-PAGE and immunoblotting procedures performed in this study were described previously [15]. Briefly, protein extracts were fractionated by SDS-PAGE according to the protocol of Laemmli [28]. The fractionated proteins were electrophoretically transferred to nitrocellulose paper [29] and probed with a primary antibody diluted as indicated in 1 × PTX solution [0.01 M phosphate, pH 7.5, 0.2% Triton X-100, 0.15 M NaCl, 1 mM EGTA, 4% BSA]. Primary antibody preparations used in this study included two BP sera, BP-R1 and BP-C1, and a rabbit antiserum, R22-31 [15], directed against the fusion protein encoded by clone λBP180-22A. Bound antibodies were detected using ^{125}I Staphylococcus aureus protein A. Immunoreactive protein bands were visualized by autoradiography using Kodak XAR5 film with DuPont Lightning Plus intensifying screens.

RESULTS

Isolation and Characterization of BP180 cDNA Clones The initial BP180 cDNA clone (clone 22-A) was identified on the basis of reactivity of its product with BP autoantibodies [15]. Sequential nucleotide screening of two independent human keratinocyte cDNA libraries has now led to the isolation of a set of overlapping cDNA clones spanning 4,669 bases of the BP180 transcript. Figure 1 shows the alignment of these clones and the position and orientation of the oligonucleotide primers used for sequencing.

Polymerase chain reaction (PCR) analysis was used to confirm the contiguity of the cDNA clones shown in Fig 1. Overlapping segments spanning 4,386 bp of the BP180 transcript were PCR-amplified from a human keratinocyte cDNA library using BP180 specific primers. The library that was used as the PCR template was unrelated to the libraries from which the BP180 clones were isolated. Figure 2 shows the results of this analysis. In each case a PCR product of the predicted size was obtained.

Sequence Analysis The entire length of the negative strand and 90% of the positive strand of the cloned portion of BP180 was sequenced by the dideoxynucleotide chain termination procedure. All G-C-rich regions that exhibited compression artifacts on the sequencing gels were sequenced by substituting either dITP or 7-deaza-dGTP in place of dGTP. The identification of the positive and negative strands was based on previously reported Northern blot analyses using strand-specific cRNA probes [15]. This cloned portion of the BP180 transcript was found to contain one long open reading frame (ORF) in the sense orientation extending from nucleotide 1 to the in-frame stop codon at position 4597–4599. This ORF encodes a polypeptide with a calculated molecular weight of 155,000 daltons and an isoelectric point of 9.7. The BP180 nucleotide and deduced amino acid sequence is shown in Fig 3.

An analysis of the deduced amino acid sequence of BP180 revealed several interesting structural features. The C-terminal half of this polypeptide, a stretch of 916 amino acids, is made up primarily of tandem repeats of the tripeptide, Glycine-X-Y, with a very high proline content at the X and Y positions. This sequence pattern is highly predictive of a protein domain that participates in the formation of a collagen triple helix. Interspersed within this long collagenous region are 15 stretches of non-collagen sequence, with lengths of 6 to 58 residues, demarcating 15 collagen domains (ranging in size from 15 to 242 amino acids; designated by the shaded areas in Fig 3). Seventy-six amino acids upstream of the collagenous region is a putative membrane-spanning domain (position 502–524), which is flanked by an upstream lysine and two downstream glutamate residues. Upstream of this potential transmembrane domain are four closely spaced cysteine residues and a 25-residue stretch containing 15 glycines and five serines. Located at position 261–358 are four tandemly arranged 24 residue repeat sequences. The alignment of these repeats is shown in Fig 4. The stretch from amino acid 1 to 229 of this cloned portion of BP180 has a particularly high isoelectric point of 11.34. A schematic representation of the structural domain organization of BP180 is shown in Fig 5.

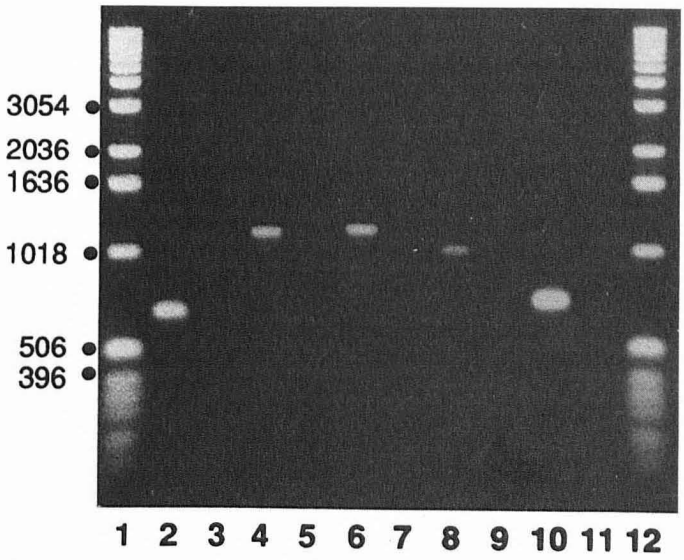

Figure 2. Polymerase chain reaction analysis. This analysis was performed to confirm the contiguity of the series of overlapping BP180 cDNA clones that are represented in Fig 1. Synthetic oligonucleotide primers based on the sequence obtained from the BP180 clones were used to amplify segments of the BP180 transcript from a human keratinocyte cDNA library. The library that was used as the PCR template is not related to the libraries from which the BP180 clones were isolated. The PCR reaction products are shown in *lanes 2, 4, 6, 8,* and *10* and the corresponding "no template" controls are shown in *lanes 3, 5, 7, 9,* and *11*. The PCR primers (designated by the nucleotide number of the primer's 5′ terminus and by the suffix "neg" or "pos" indicating the negative or positive strand, respectively) were used in the following combinations: 37pos and 707neg (*lanes 2* and *3*); 538pos and 1701neg (*lanes 4* and *5*); 1645pos and 2824neg (*lanes 6* and 7); 2807pos and 3834neg (*lanes 8* and *9*); 3690pos and 4423neg (*lanes 10* and *11*). The predicted sizes of the PCR products are 670 bp, *lane 2*; 1163 bp, *lane 4*; 1179 bp, *lane 6*; 1027 bp, *lane 8*; 733 bp, *lane 10*. On the left of the gel are the lengths (in bp) of the DNA size markers (1 kb ladder, BRL) that were run in *lanes 1* and *12*.

The BP180 sequence was compared with the GenBank nucleotide sequence database (release 69) and the Swiss protein sequence database (release 19). As expected, homologies were observed with all members of the collagen family. Human collagens α1(I), α1(III), α2(IV), α2(XI), and α1(XI) showed similarities of 56%, 55%, 53%, 53%, and 52%, respectively, over stretches of approximately 1100 amino acids. A particularly high degree of sequence homology, at both the nucleotide and amino acid levels, was detected between BP180 and a chick cornea collagen recently reported by Marchant et al [30]. The amino acid sequence alignment of these two polypeptides, shown in Fig 6, reveals identical residues at 64% of the positions with an additional 14% of the positions containing conservative substitutions. In addition to the overall sequence similarities between these two polypeptides, there is a striking conservation in the sizes and positions of their collagen domains (indicated by the shaded boxes in Fig 6). Comparison of the BP180 cDNA sequence with that of the previously published BP230 cDNA [19] revealed no significant homology at either the nucleotide or amino acid levels.

Collagenase Digestions To further document the existence of a collagenous structure within the BP180 epidermal autoantigen, a human skin extract was digested with bacterial collagenase under various conditions and analyzed by SDS-PAGE and immunoblotting with a BP serum, BP-R1, and a rabbit antiserum, R22-31, directed against the βgal-BP180A fusion protein [15]. At time zero the epidermal extract was shown to contain BP180 as a 180-kD antigen recognized by serum BP-R1 (Fig 7, *lane 1*). *Lanes 2, 3,* and *4*

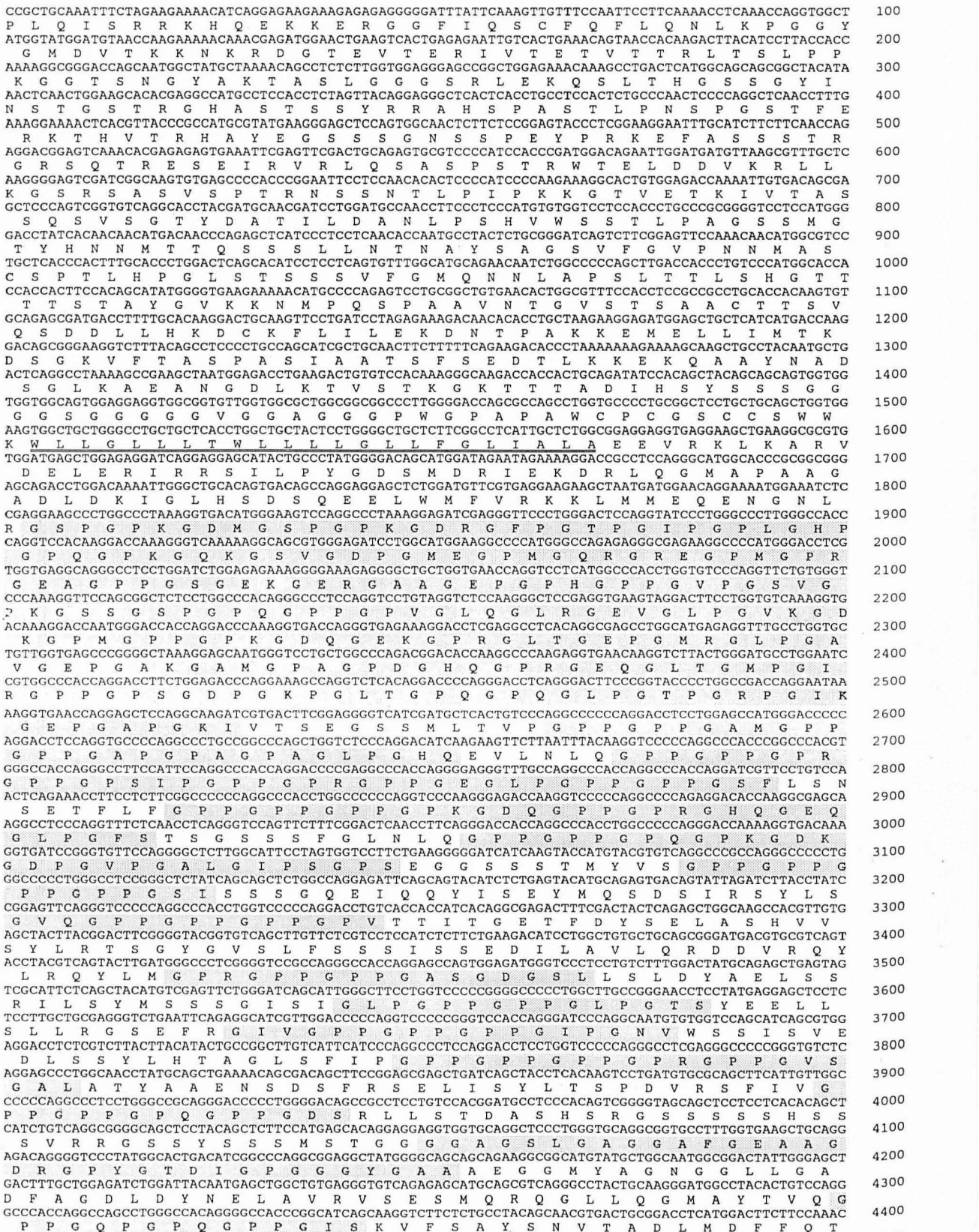

Figure 3. Nucleotide and deduced amino acid sequence of the human BP180 autoantigen. This nucleotide sequence, obtained from the set of overlapping BP180 cDNA clones represented in Fig 1, contains one long open reading frame encoding a polypeptide of 155 kD with an isoelectric point of 9.7. The putative transmembrane domain is indicated by the *double underline*. *Shaded areas*, collagen triple helical domains. The stop codon is marked with an *asterisk*. This sequence appears to be missing the 5′ terminus of the BP180 coding region.

of Fig 7 show the immunoblot analysis of this epidermal extract after 10 min, 30 min, and 2 h of collagenase digestion, respectively. This time-course analysis shows the gradual disappearance of the 180-kD band, the transient appearance of a 102-kD band, and finally a 97-kD collagenase-resistant polypeptide. All three of these bands exibited reactivity with serum BP-R1, indicating that the two smaller polypeptides arose as a breakdown of the full-length BP180 antigen. As further verification of the relationship between the 180-kD and the 97-kD collagenase-resistant bands, rabbit antiserum, R22-31, was shown to react on immunoblot analysis with both of these polypeptides (Fig 7, *lanes 6* and 7). A variety of controls were used, as described in *Materials and Methods*, to demonstrate that, under the conditions of this assay, degradation of BP180 was due to the action of the well-characterized Ca^{++}-dependent bacterial collagenase, and not due to the action of contaminating proteolytic activity. For example, replacement of the $CaCl_2$ with EDTA in the reaction mixture resulted in an inhibition of digestion of the BP180 epidermal antigen even after a 2-h incubation at 37°C with 1 BTC unit of collagenase (Figure 7, lane 5). This result rules out the action of a Ca^{++}-independent protease as a mechanism of BP180 degradation in our assay. Immunoblot analysis of this epidermal extract with a second BP serum, BP-C1 (which reacts strongly with the BP230 antigen) revealed that BP230 remained intact after a 2-h treatment with 1 BTC unit of collagenase at 37°C in the presence of buffer TBSC, which contains 10 mM $CaCl_2$ (data not shown). Positive and negative controls included PAGE analysis of collagenase digestions of type I calf skin collagen, BSA, and bovine muscle myosin.

DISCUSSION

Circulating autoantibodies from BP patients have previously been shown to bind hemidesmosomal antigens of squamous epithelia [4–7] and to react, by immunoblotting and immunoprecipitation, with two large epidermal proteins, designated as the BP230 and BP180 antigens [10–12]. Our laboratory has previously reported the characterization of a human epidermal cDNA encoding a portion of the BP180 autoantigen using a human BP serum, BP-R1 [15]. The present report describes the isolation and sequence analysis of a series of overlapping cDNA clones that spans 4,669 bases of the BP180 transcript.

The contiguity of the BP180 cDNA was established using several lines of evidence. First, the cDNA stretch corresponding to nucleotide numbers 1 to 2679 is represented in multiple independent clones. Second, the cDNA stretch corresponding to nucleotide numbers 1312 to 4600 shows a high degree of homology, both at the nucleotide and the amino acid levels, with a chick cornea cDNA sequence reported by Marchant et al [30]. Finally, the contiguity of the BP180 clones was confirmed by PCR analysis. Using BP180-specific primers, overlapping cDNA segments spanning 4,387 bases of the BP180 transcript were PCR-amplified from a cDNA library unrelated to the libraries from which the original set of BP180 clones was isolated.

This cloned portion of BP180 was shown to contain one long open reading frame encoding a 155-kD polypeptide. It is not yet clear whether this stretch encompasses the entire BP180 coding region. Contained within this cloned segment was an in-frame stop codon at position 4597–4599. The first in-frame AUG codon appears at position 106–108; however, this is not likely to be the translation initiation site because of the presence of an out-of-frame AUG sequence at position 101–103. Repeated attempts to isolate additional cDNA clones containing sequences upstream of our 5′-most clone, clone 262-F, have been unsuccessful. Identification of the N-terminus of BP180 via protein microsequencing has thus far been precluded due to the lack of an optimal source for the isolation of sufficient quantities of this protein.

Analysis of the BP180-deduced amino acid sequence identified a single, putative membrane-spanning domain at amino acid position 502–524, which has the potential to function as a signal-anchor sequence for a type II transmembrane protein (see below). The polypeptide segment on the N-terminal side of this putative transmembrane domain is highly basic (pI of this 501 amino acid stretch is 10.37, whereas the pI of amino acids 1–229 is even higher, 11.34) and was shown to contain a degenerate set of four 24–26 residue tandem repeats. The relevance of these repeats remains undetermined. Comparison of the nucleotide and amino acid sequences of this putative intracellular segment of BP180 with the entries of the GenBank and EMBL nucleotide sequence data bases and the Swiss protein sequence data base revealed no significant homologies.

The most striking structural feature of the BP180 protein is the long C-terminal collagenous region. This stretch of almost 100 kD is made up of 15 domains of variable size that are predicted to form collagen-like triple helices interrupted by segments of non-collagen sequence. We propose that the collagen domains (COL1 through COL15) and the non-collagen domains (NC1 through NC16) be numbered starting at the C-terminus. Domain NC16, which encompasses the membrane-spanning domain, has been further subdivided as follows: NC16a corresponds to that portion lying to the C-terminal side of the transmembrane domain (designated NC16b), whereas the remaining N-terminal segment is labeled NC16c (see Fig 5). This sequence analysis predicts BP180 to be a transmembrane protein with a type II orientation, i.e., the C-terminal collagenous region positioned externally, as represented schematically in Fig 5. This transmembrane orientation has been supported by recent ultrastructural localization studies using antibodies generated against two BP180 recombinant proteins corresponding to portions of the predicted cytoplasmic and ectodomains [31].

The C-terminal 1,098 amino acids of BP180 exhibit a particu-

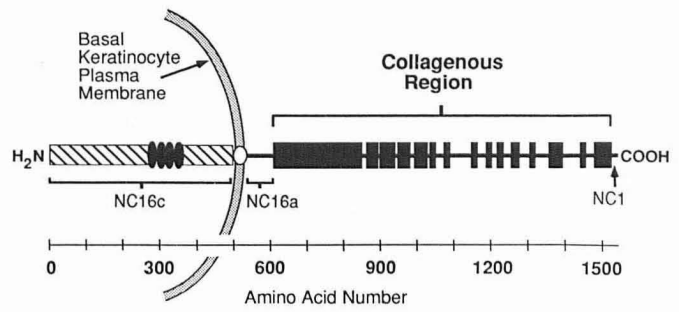

Figure 5. Schematic representation of the domain organization of the human BP180 antigen. The putative membrane-spanning domain is designated by the *open oval*. The C-terminal 1007 amino acid stretch is shown as an extracellular domain that is predicted to extend into the basal lamina region adjacent to the basal keratinocyte. This C-terminal region consists of a series of 15 collagen domains (COL1 through COL15; *solid boxes*) and stretches of non-collagen sequence (NC1 through NC16a; *thin lines*). Numbering of both the collagen and non-collagen domains starts at the C-terminus. The non-collagen domain, NC16, encompasses the membrane-spanning domain, NC16b, as well as flanking regions to the C-terminal and N-terminal sides (NC16a and NC16c, respectively). Domain NC16c, the N-terminal segment predicted to be an intracellular domain, includes the four tandem repeats (shown in Fig 4 and indicated here as *solid ovals*).

BP180 Tandem Repeats

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262  AGSSMGTYHNNMTTQSSSLLNTNAYS
288  AGSVFG-VPNNMASCSP-TLHPGLST
312  SSSVFG-MQNNLA-PSLTTLSHGTTT
336  TSTAYG-VKKNMP-QSPAAVNTGVST
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Figure 4. Alignment of BP180 tandem repeats. Four contiguous stretches of the BP180 protein sequence from residue 262 through 359 exhibit significant sequence similarities. *Shaded areas*, positions of identity or conservative substitutions, as defined by Bordo and Argos [41].

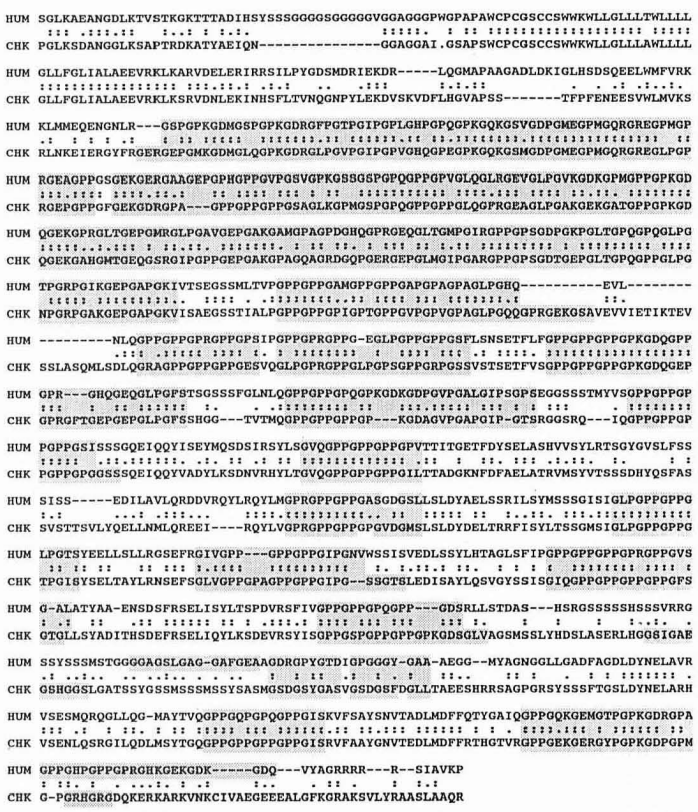

Figure 6. Alignment of the amino acid sequences deduced from the human BP180 cDNA and a chick cornea collagen cDNA [30]. The region of overlap corresponds to amino acid positions 435 to 1532 of the human sequence. These two sequences exhibit 64% identity and 78% similarity. Optimal alignment was obtained by the Bestfit program of the GCG software package [26]. Identical residues are indicated by a *double dot* and similar residues by a *single dot*. Note the high degree of conservation of the sizes and positions of the collagen domains designated by the *shaded boxes*.

larly high degree of similarity with a recently reported sequence obtained from a chick cornea cDNA [30]. These structural similarities strongly suggest that these two proteins are human and chicken homologues. The short gaps that were present in the human and chick protein alignment probably represent inter-species divergence; however, because these two cDNA segments originated from different tissues (human epidermis and chick cornea), the observed divergence could alternatively be due to the existence of tissue-specific BP180 isoforms. A related issue that remains to be resolved is whether the human BP180 antigen (or an isoform) is a component of the hemidesmosomes of cornea or other stratified, squamous epithelia, as has been shown to be the case in human epidermis [15]. No data have yet been reported concerning the transmembrane nature or the ultrastructural localization of the chick cornea protein.

As a means of independently demonstrating the presence of collagen-like sequences within the BP180 autoantigen, a human skin extract was treated with collagenase from *Clostridium histolyticum* and the digestion products were subsequently analyzed by SDS-PAGE and immunoblot. Detailed characterization of the substrate specificities of the collagenases from this bacterium has been previously reported [32–35], and has allowed for the identification of potential collagenase-sensitive sites on BP180. Based on this analysis it was predicted that complete collagenase digestion of the BP180 antigen would result in the degradation of the C-terminal 87-kD segment of this protein to small peptide fragments. The experimental results coincided quite well with this prediction. Collagenase treatment of the epidermal extract resulted in specific degradation of approximately 83 kD of the BP180 antigen, generating one major collagenase-resistant, autoantibody-reactive peptide of 97 kD. These results verify the existence of a large collagenous region on the 180-kD human epidermal BP antigen.

The results of the above immunoblot analysis also demonstrate that an epitope recognized by BP-R1 autoantibodies is located within the 97-kD collagenase-resistant fragment of BP180. This finding is consistent with results previously reported by our group [36]. In this earlier study a BP autoantibody-reactive epitope was shown to be located within the major collagenase-resistant fragment of the protein encoded by clone λBP180-22A. The epitope-bearing segment of this recombinant form of BP180, a stretch that is also predicted to be present within the 97 kD collagenase-resistant BP180 fragment, has recently been further characterized by epitope mapping and ultrastructural immunolocalization analyses. A major antigenic site recognized by autoantibodies from BP patients, as well as from patients suffering from herpes gestationis (a clinically related subepidermal dermatosis of pregnancy), was shown to be restricted to a 14 amino acid segment located in the NC16a domain (between the putative transmembrane domain and the collagenous

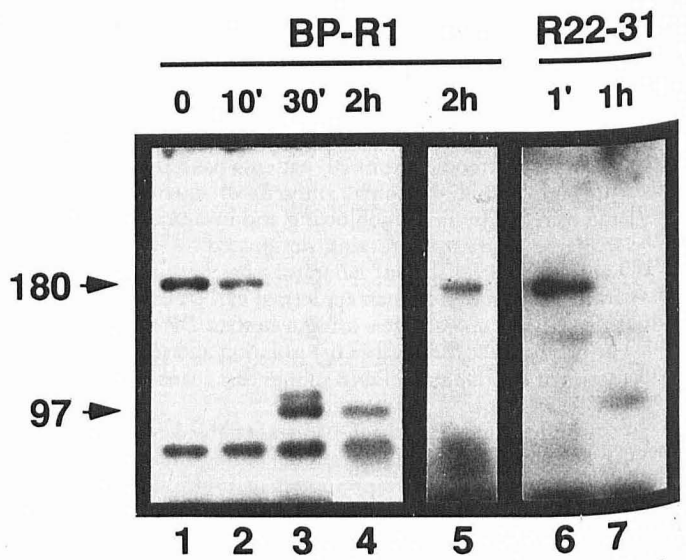

Figure 7. Immunoblot analysis of a human epidermal extract treated with bacterial collagenase. A human epidermal extract containing the BP180 antigen was subjected to collagenase digestion and the products were fractionated by SDS-PAGE, blotted to nitrocellulose, and labeled with either serum BP-R1, a well-characterized BP serum that recognizes BP180 (*lanes 1–5*), or with a rabbit antiserum, R22-31, which was prepared against the β-galactosidase-BP180A fusion protein (*lanes 6* and 7). The *numbers* to the left of the figure indicate apparent molecular weight (in kD). *Lanes 1* through *4* show the results of a time course of digestion with 1 BTC unit of collagenase at 37°C in the presence of 10 mM $CaCl_2$ for the length of time indicated at the top of each *lane* (0, 10 min, 30 min, and 2 h, respectively). BP180, identified as the 180-kD antigen in the untreated extract, undergoes digestion to a 102-kD intermediate (*lane 3*) and a 97-kD fragment that remains after complete collagenase digestion (2-h time point, *lane 4*). All three bands (180 kD, 102 kD, and 97 kD) exhibited reactivity with serum BP-R1. An immunoreactive band with an apparent molecular weight of 82 kD is seen in *lanes 1* through *4*. This polypeptide, which was unaffected by the collagenase treatment, is of unknown relevance. *Lane 5* shows the immunoblot result of the same epidermal extract after a 2-h collagenase treatment in the presence of 75 mM EDTA (an inhibitor of the bacterial collagenase). No digestion of BP180 was detected. *Lanes 6* and 7 represent immunoblots of the epidermal extract treated with collagenase in the presence of $CaCl_2$ for 1 min and 60 min, respectively, and subsequently labeled with rabbit antiserum, R22-31. This antiserum recognizes full-length BP180 (*lane 6*) and the 97-kD collagenase-resistant BP180 fragment (*lane 7*).

region)* [37]. Furthermore, these studies have localized this epitope-bearing domain of BP180 to the extracellular region of the epidermal hemidesmosome* [31]. These results are consistent with our predicted type II transmembrane orientation of BP180.

Based on the structural analysis presented in this report, BP180 is predicted to be an integral membrane protein that, in the simplest case, associates with two other polypeptides to form an extracellular collagen triple helix. This remarkable combination of structural features has only been found in two other proteins, the type I and type II macrophage scavenger receptors [38,39]. Both of these closely related proteins have a single transmembrane domain with the same uncommon type II orientation predicted for BP180. Such proteins have a signal-anchor sequence in lieu of an amino-terminal signal peptide [40]. The extracellular region of both forms of the macrophage receptor contains a coiled coil domain and a collagen triple helical domain. In addition, this C-terminal ectodomain has been shown to function in cell-ligand interactions [39]. More detailed analyses reveal limitations in the structural similarities between BP180 and the macrophage receptors. These proteins share no significant sequence homology except for the regularly spaced glycine residues within the collagenous regions. The NC16a domain of BP180, the segment that corresponds in position to the coiled coil domain of the macrophage scavenger receptors, has only a very short stretch with the primary structural features consistent with a coiled coil structure.

In summary, this molecular analysis of the BP180 antigen has revealed several structural features that suggest that this protein may function as a cell-matrix adhesion molecule. BP180 appears to be an integral membrane protein with a localization pattern restricted to the hemidesmosomes of stratified, squamous epithelia. The carboxyl-terminal half of BP180 is made up of multiple domains that contain the basic structural requirements for forming collagen-like triple helices. This collagenous region of BP180 is predicted to be an extracellular domain that may function as a site of interaction with an extracellular matrix component, stabilizing the dermal-epidermal association. Disruption of such an adhesive interaction, via either autoantibody reactivity (as in the case of BP or HG) or genetic mutation (as in certain genodermatoses, such as junctional epidermolysis bullosa), may play a critical role in the pathogenesis of these sub-epidermal blistering disorders.

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